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University of Ulster
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Patents ADP number (if you know it)

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7384134001

4. Title of the invention

"Peptide"

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Murgitroyd & Company

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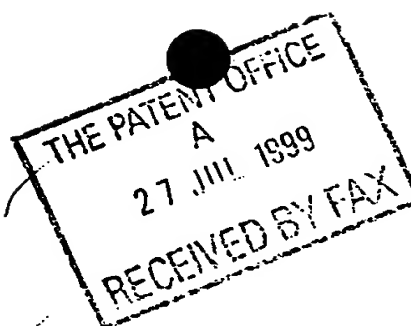
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Abstract

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1

1 "PEPTIDE"

2

3 The present invention relates to release of insulin and
4 control of blood glucose concentration. More
5 particularly the invention relates to the use of
6 peptides to stimulate release of insulin, lowering of
7 blood glucose and pharmaceutical preparations for
8 treatment of type 2 diabetes.

9

10 Gastric inhibitory polypeptide (GIP) and glucagon-like
11 peptide-1 (7-36)amide (truncated GLP-1; tGLP-1) are two
12 important insulin-releasing hormones secreted from
13 endocrine cells in the intestinal tract in response to
14 feeding [1,2]. Together with autonomic nerves they
15 play a vital supporting role to the pancreatic islets
16 in the control of blood glucose homeostasis and
17 nutrient metabolism [1,3].

18

19 Dipeptidyl peptidase IV (DPP IV; EC 3.4.14.5) has been
20 identified as a key enzyme responsible for inactivation
21 of GIP and tGLP-1 in serum [4,5]. DPP IV is completely
22 inhibited in serum by the addition of diprotin A (DPA,
23 0.1 mmol/l) [4]. This occurs through the rapid removal
24 of the N-terminal dipeptides Tyr¹-Ala² and His⁷-Ala⁸
25 giving rise to the main metabolites GIP(3-42) and GLP-

1 1(9-36)amide, respectively. These truncated peptides
2 are reported to lack biological activity or to even
3 serve as antagonists at GIP or tGLP-1 receptors [6-9].
4 The resulting biological half-lives of these incretin
5 hormones in vivo are therefore very short, estimated to
6 be no longer than 5 min [5, 10-12].

7

8 In situations of normal glucose regulation and
9 pancreatic B-cell sensitivity, this short duration of
10 action is advantageous in facilitating momentary
11 adjustments to homeostatic control. However, the
12 current goal of a possible therapeutic role of incretin
13 hormones, particularly tGLP-1 in NIDDM therapy is
14 frustrated by a number of factors in addition to
15 finding a convenient route of administration [13].
16 Most notable of these are rapid peptide degradation and
17 rapid absorption (peak concentrations reached 20 min)
18 and the resulting need for both high dosage and precise
19 timing with meals [13-15]. Recent therapeutic
20 strategies have focused on precipitated preparations to
21 delay peptide absorption [16] and inhibition of GLP-1
22 degradation using specific inhibitors of DPP IV [17-
23 19]. A possible therapeutic role is also suggested by
24 the observation that a specific inhibitor of DPP IV,
25 isoleucine thiazolidide, lowered blood glucose and
26 enhanced insulin secretion in glucose-treated diabetic
27 obese Zucker rats presumably by protecting against
28 catabolism of the incretin hormones tGLP-1 and GIP
29 [18].

30

31 Numerous studies have indicated that tGLP-1 infusion
32 restores pancreatic B-cell sensitivity, insulin
33 secretory oscillations and improved glycemic control in
34 various groups of patients with IGT or NIDDM [13,15,20-
35 22]. Longer term studies also show significant
36 benefits of tGLP-1 injections in NIDDM and possibly

1 IDDM therapy [20,23,24], providing a major incentive to
2 develop an orally effective or long-acting tGLP-1
3 analogue [13]. Several attempts have been made to
4 produce structurally modified analogues of tGLP-1 which
5 are resistant to DPP IV degradation [25-27]. A
6 significant extension of serum half-life is observed
7 with His⁷- glucitol tGLP-1 and tGLP-1 analogues
8 substituted at position 8 with Gly, Aib, Ser or Thr
9 [25-27]. However, these structural modifications seem
10 to impair receptor binding and insulinotrophic activity
11 thereby compromising part of the benefits of protection
12 from proteolytic degradation [25-28]. In recent
13 studies using His⁷-glucitol tGLP-1, resistance to DPP IV
14 and serum degradation was accompanied by severe loss of
15 insulin-releasing activity [26,28].

16
17 GIP shares not only the same degradation pathway as
18 tGLP 1 but many similar physiological actions,
19 including stimulation of insulin and somatostatin
20 secretion, and the enhancement of glucose disposal [1].
21 These actions are viewed as key aspects in the
22 antihyperglycemic properties of tGLP-1 [13], and there
23 is therefore good expectation that GIP may have similar
24 potential as NIDDM therapy. Indeed, compensation by
25 GIP is held to explain the modest disturbances of
26 glucose homeostasis observed in tGLP-1 knockout mice
27 [29]. Apart from early studies [30], the anti-diabetic
28 potential of GIP has not been explored and tGLP-1 may
29 seem more attractive since it is viewed by some as a
30 more potent insulin secretagogue when infused at "so
31 called" physiological concentrations estimated by RIA
32 [31].

33
34 It has been shown that N-terminal glycation of GIP
35 markedly enhances the insulin releasing effect of the
36 peptide on clonal B-cells [32].

The present invention aims to provide effective analogues of GIP. It is one aim of the invention to provide a pharmaceutical for treatment of Type 2 diabetes.

According to the present invention there is provided effective analogues of the biologically active GIP(1-42) which have improved characteristics for treatment of Type 2 diabetes wherein the analogues have amino acid substitutions at positions 1-3 and or contain various other amino acid substitutions in the basic peptide. The structures of human and porcine GIP(1-42) are shown below. The porcine peptide differs by just two amino acid substitutions at positions 18 and 34.

Fig. 1. Primary structure of human gastric inhibitory polypeptide (GIP)

1	5	10	15	20	25
NH ₂ -Tyr-Ala-Glu-Gly-Thr-Phe-Ile-Ser-Asp-Tyr-Ser-Ile-Ala-Met-Asp-Lys-Ile-His-Gln-Gln-Asp-Phe-Val-Asn-Trp-					
Leu-Leu-Ala-Gln-Lys-Gly-Lys-Lys-Asn-Asp-Trp-Lys-His-Asn-Ile-Thr-Gln-COOH					
	30	35	40		

Fig. 2. Primary structure of porcine gastric inhibitory polypeptide (GIP)

1	5	10	15	20	25
NH ₂ -Tyr-Ala-Glu-Gly-Thr-Phe-Ile-Ser-Asp-Tyr-Ser-Ile-Ala-Met-Asp-Lys-Ile-Arg-Gln-Gln-Asp-Phe-Val-Asn-Trp-					
Leu-Leu-Ala-Gln-Lys-Gly-Lys-Lys-Ser-Asp-Trp-Lys-His-Asn-Ile-Thr-Gln-COOH					
	30	35	40		

The analogues of GIP(1-42) may have an enhanced capacity to stimulate insulin secretion, enhance glucose disposal, delay glucose absorption or may exhibit enhanced stability in plasma as compared to native GIP.

Any of these properties will enhance the potency of the analogue as a therapeutic agent.

1 Analogues having D-amino acid substitutions in the 1, 2
2 and 3 positions and/or N-glycated, N-alkylated, N-
3 acetylated or N-acylated amino acids in the 1 position
4 are resistant to degradation in vivo.

5
6 Various amino acid substitutions at second and third
7 amino terminal residues are included, such as GIP(1-
8 42)Gly2, GIP(1-42)Ser2, GIP(1-42)Abu2, GIP(1-42)Aib,
9 GIP(1-42)D-Ala2, GIP(1-42)Sar2, and GIP(1-42)Pro3.

10
11 Amino-terminally modified GIP analogues include N-
12 glycated GIP(1-42), N-alkylated GIP(1-42), N-acetylated
13 GIP(1-42), N-acetyl-GIP(1-42) and N-isopropyl GIP(1-
14 42).

15
16 Other stabilised analogues include those with a peptide
17 isostere bond between amino terminal residues at
18 position 2 and 3. These analogues may be resistant to
19 the plasma enzyme dipeptidyl-peptidase IV (DPP IV)
20 which is largely responsible for inactivation of GIP by
21 removal of the amino-terminal dipeptide Tyr1-Ala2.

22
23 In particular embodiments, the invention provides a
24 peptide which is more potent than human or porcine GIP
25 in moderating blood glucose excursions, said peptide
26 consisting of GIP(1-42) or N-terminal fragments of
27 GIP(1-42) consisting of up to 30 amino acid residues
28 from the N-terminus (i.e. GIP(1-30)) with one or more
29 modifications selected from the group consisting of:

- 30
31 (a) substitution of Ala2 by Gly
32 (b) substitution of Ala2 by Ser
33 (c) substitution of Ala2 by Abu
34 (d) substitution of Ala2 by Aib
35 (e) substitution of Ala2 by D-Ala
36 (f) substitution of Ala2 by Sar

- (g) substitution of Glu³ by Pro
- (h) modification of Tyr¹ by acetylation
- (i) modification of Tyr¹ by acylation
- (j) modification of Tyr¹ by alkylation
- (k) modification of Tyr¹ by glycation
- (l) conversion of Ala²-Glu³ bond to a psi [CH₂NH] bond
- (m) conversion of Ala²-Glu³ bond to a stable peptide isostere bond
- (n) (n-isopropyl-H) 1GIP.

A preferred embodiment of the invention provides the use of Tyr¹-glucitol GIP in the preparation of a medicament for the treatment of diabetes.

The invention further provides improved pharmaceutical compositions including analogues of GIP with improved pharmacological properties.

Other possible analogues include certain commonly encountered amino acids, which are not encoded by the genetic code, for example, beta-alanine (beta-ala), or other omega-amino acids, such as 3-amino propionic, 4-amino butyric and so forth, alpha-aminoisobutyric acid (Aib), sarcosine (Sar), ornithine (Orn), citrulline (Cit), homoarginine (Har), t-butylalanine (t-BuA), t-butylglycine (t-BuG), N-methylisoleucine (N-MeIle), phenylglycine (Phg), and cyclohexylalanine (Cha), norleucine (Nle), cysteic acid (Cya) and methionine sulfoxide (MSO), substitution of the D form of a neutral or acidic amino acid or the D form of tyrosine for tyrosine.

According to the present invention there is also provided a pharmaceutical composition useful in the treatment of diabetes type II which comprises an effective amount of the peptide as described herein, in

admixture with a pharmaceutically acceptable excipient.

The invention will now be demonstrated with reference to the following non-limiting example and the accompanying figures wherein:

Figure 1 illustrates degradation of GIP and Tyr¹-glucitol GIP by DPP IV.

Figure 2 illustrates degradation of GIP and Tyr¹-glucitol GIP by human plasma.

Figure 3 illustrates electrospray ionization mass spectrometry of GIP, Tyr¹-glucitol GIP and the major degradation fragment GIP(3-42).

Figure 4 shows the effects of GIP and glycated GIP on plasma glucose homeostasis.

Figure 5 shows Effects of GIP on plasma insulin responses (A).

Example

The following example investigates preparation of Tyr¹-glycitol GIP together with evaluation of its antihyperglycemic and insulin-releasing properties in vivo. The results clearly demonstrate that this novel GIP analogue extracts a substantial resistance to aminopeptidase degradation and increased glucose lowering activity compared with the native GIP.

Research Design and Methods

Materials. Human GIP was purchased from the American Peptide Company (Sunnyvale, CA, USA). HPLC grade

acetonitrile was obtained from Rathburn (Walkersburn, Scotland). Sequencing grade trifluoroacetic acid (TFA) was obtained from Aldrich (Poole, Dorset, UK). All other chemicals purchased including dextran T-70, activated charcoal, sodium cyanoborohydride and bovine serum albumin fraction V were from Sigma (Poole, Dorset, UK). Diprotin A (DPA) was purchased from Calbiochem-Novabiochem (UK) Ltd. (Beeston, Nottingham, UK) and rat insulin standard for RIA was obtained from Novo Industria (Copenhagen, Denmark). Reversed-phase Sep-Pak cartridges (C-18) were purchased from Millipore-Waters (Milford, MA, USA). All water used in these experiments was purified using a Milli-Q, Water Purification System (Millipore Corporation, Milford, MA, USA).

Preparation of Tyr¹-glucitol GIP. Human GIP was incubated with glucose under reducing conditions in 10 mmol/l sodium phosphate buffer at pH 7.4 for 24 h. The reaction was stopped by addition of 0.5 mol/l acetic acid (30 μ l) and the mixture applied to a Vydac (C-18) (4.6 x 250mm) analytical HPLC column (The Separations Group, Hesperia, CA, USA) and gradient elution conditions were established using aqueous/TFA and acetonitrile/TFA solvents. Fractions corresponding to the glycosylated peaks were pooled, taken to dryness under vacuum using an AES 1000 Speed-Vac concentrator (Life Sciences International, Runcorn, UK) and purified to homogeneity on a Supelcosil (C-8) (4.6 x 150mm) column (Supelco Inc., Poole, Dorset, UK).

Degradation of GIP and Tyr¹-glucitol GIP by DPP IV. HPLC-purified GIP or Tyr¹-glucitol GIP were incubated at 37°C with DPP-IV (5mU) for various time periods in a reaction mixture made up to 500 μ l with 50 mmol/l triethanolamine-HCl, pH 7.8 (final peptide

concentration 1 $\mu\text{mol/l}$) [4]. Enzymatic reactions were terminated after 0, 2, 4 and 12 hours by addition of 5 μl of 10% (v/v) TFA/water. Samples were made up to a final volume of 1.0 ml with 0.12% (v/v) TFA and stored at -20°C prior to HPLC analysis.

Degradation of GIP and Tyr¹-glucitol GIP by human plasma. Pooled human plasma (20 μl) taken from six healthy fasted human subjects was incubated at 37°C with GIP or Tyr¹-glucitol GIP (10 μg) for 0 and 4 hours in a reaction mixture made up to 500 μl , containing 50 mmol/l triethanolamine/HCL buffer pH 7.8. Incubations for 4 hours were also performed in the presence of diprotin A (5 mU). The reactions were terminated by addition of 5 μl of TFA and the final volume adjusted to 1.0 ml using 0.1% v/v TFA/water. Samples were centrifuged (13,000g, 5 min) and the supernatant applied to a C-18 Sep-Pak cartridge (Millipore-Waters) which was previously primed and washed with 0.1% (v/v) TFA/water. After washing with 20 ml 0.12% TFA/water, bound material was released by elution with 2 ml of 80% (v/v) acetonitrile/water and concentrated using a Speed-Vac concentrator (Runcorn, UK). The volume was adjusted to 1.0ml with 0.12% (v/v) TFA/water prior to HPLC purification.

HPLC analysis of degraded GIP and Tyr¹-glucitol GIP. Samples were applied to a Vydac C-18 widepore column equilibrated with 0.12% (v/v) TFA/H₂O at a flow rate of 1.0 ml/min. Using 0.1% (v/v) TFA in 70% acetonitrile/H₂O, the concentration of acetonitrile in the eluting solvent was raised from 0% to 31.5% over 15 min, to 38.5% over 30 min and from 38.5% to 70% over 5 min, using linear gradients. The absorbance was monitored at 206 nm and peak areas evaluated using a model 2221 LKB integrator. Samples recovered manually

1 were concentrated using a Speed-Vac concentrator.

2
3 **Electrospray ionization mass spectrometry (ESI-MS).**

4 Samples for ESI-MS analysis containing intact and
5 degradation fragments of GIP (from DPP IV and plasma
6 incubations) as well as Tyr¹-glucitol GIP, were further
7 purified by HPLC. Peptides were dissolved
8 (approximately 400 pmol) in 100 µl of water and applied
9 to the LCG benchtop mass spectrometer (Finnigan MAT,
10 Hemel Hempstead, UK) equipped with a microbore C-18
11 HPLC column (150 x 2.0mm, Phenomenex, UK, Ltd,
12 Macclesfield). Samples (30µl direct loop injection)
13 were injected at a flow rate of 0.2ml/min, under
14 isocratic conditions 35% (v/v) acetonitrile/water. Mass
15 spectra were obtained from the quadripole ion trap mass
16 analyzer and recorded. Spectra were collected using
17 full ion scan mode over the mass-to-charge (m/z) range
18 150-2000. The molecular masses of GIP and related
19 structures were determined from ESI-MS profiles using
20 prominent multiple charged ions and the following
21 equation $M_x = iM_1 - iM_h$ (where M_x = molecular mass; M_1 =
22 m/z ratio; i = number of charges; M_h = mass of a
23 proton).
24

25 **In vivo biological activity of GIP and Tyr¹-glucitol**
26 **GIP.** Effects of GIP and Tyr¹-glucitol GIP on plasma
27 glucose and insulin concentrations were examined using
28 10-12 week old male Wistar rats. The animals were
29 housed individually in an air conditioned room and
30 22±2°C with a 12 hour light/12 hour dark cycle.
31 Drinking water and a standard rodent maintenance diet
32 (Trouw Nutrition, Belfast) were supplied *ad libitum*.
33 Food was withdrawn for an 18 hour period prior to
34 intraperitoneal injection of glucose alone (18mmol/kg
35 body weight) or in combination with either GIP or Tyr¹-
36 glucitol GIP (10 nmol/kg). Test solutions were

11

1 administered in a final volume of 8 ml/kg body weight.
2 Blood samples were collected at 0, 15, 30 and 60
3 minutes from the cut tip of the tail of conscious rats
4 into chilled fluoride/heparin microcentrifuge tubes
5 (Sarstedt, Nümbrecht, Germany). Samples were
6 centrifuged using a Beckman microcentrifuge for about
7 30 seconds at 13,000 g. Plasma samples were aliquoted
8 and stored at -20°C prior to glucose and insulin
9 determinations. All animal studies were done in
10 accordance with the Animals (Scientific Procedures) Act
11 1986.

12
13 Analytes. Plasma glucose was assayed by an automated
14 glucose oxidase procedure using a Beckman Glucose
15 Analyzer II [33]. Plasma insulin was determined by
16 dextran charcoal radioimmunoassay as described
17 previously [34]. Incremental areas under plasma
18 glucose and insulin curves (AUC) were calculated using
19 a computer program (CAREA) employing the trapezoidal
20 rule [35] with baseline subtraction. Results are
21 expressed as mean \pm SEM and values were compared using
22 the Student's unpaired t-test. Groups of data were
23 considered to be significantly different if $P < 0.05$.

24 25 Results

26
27 Degradation of GIP and Tyr¹-glucitol GIP by DPP IV.
28 Figure 1 illustrates the typical peak profiles obtained
29 from the HPLC separation of the products obtained from
30 the incubation of GIP (left panels) or Tyr¹-glucitol GIP
31 (right panels) with DPP IV for 0, 2, 4 and 12 hours.
32 The retention times of GIP and Tyr¹-glucitol GIP at t=0
33 were 21.93 minutes and 21.75 minutes respectively.
34 Degradation of GIP was evident after 4 hours incubation
35 (54% intact), and by 12 hours the majority (60% of
36 intact GIP was converted to the single product with a

retention time of 21.61 minutes. Tyr¹-glucitol GIP remained almost completely intact throughout 2-12 hours incubation.

Degradation of GIP and Tyr¹-glucitol GIP by human plasma. Figure 2 shows a set of typical HPLC profiles of the products obtained from the incubation of GIP or Tyr¹-glucitol GIP with human plasma for 0 and 4 h. GIP (left panels) with a retention time of 22.06 min was readily metabolised by plasma within 4 hours incubation giving rise to the appearance of a major degradation peak with a retention time of 21.74 minutes. In contrast, the incubation of Tyr¹-glucitol GIP under similar conditions (right panels) did not result in the formation of any detectable degradation fragments during this time with only a single peak being observed with a retention time of 21.77 minutes. Addition of diprotin A, a specific inhibitor of DPP IV, to GIP during the 4 hours incubation completely inhibited degradation of the peptide by plasma.

Identification of GIP degradation fragments by ESI-MS. Figure 3 shows the monoisotopic molecular masses obtained for GIP, (panel A), Tyr¹-glucitol GIP (panel B) and the major plasma degradation fragment of GIP (panel C) using ESI-MS. The peptides analyzed were purified from plasma incubations as shown in Figure 2. The exact molecular mass (M_r) of the peptides were calculated using the equation $M_r = iM_i - iM_h$ as defined in Research Design and Methods section. After spectral averaging was performed, prominent multiple charges species $(M+3H)^{3+}$ and $(M+4H)^{4+}$ were detected from GIP at m/z 1661.6 and 1246.8, corresponding to intact M_r 4981.8 and 4983.2 Da, respectively (Fig. 3A). Similarly, for Tyr¹-glucitol GIP $(M+4H)^{4+}$ and $(M+5H)^{5+}$ were detected at m/z 1287.7 and 1030.3, corresponding to intact

13

1 molecular masses of M^r 5146.8 and 5146.5 Da,
2 respectively (Fig. 3B). The difference between the
3 observed molecular masses of the quadruply charged GIP
4 and the N-terminally modified GIP species (163.6 Da)
5 indicated that the latter peptide contained a single
6 glucitol adduct corresponding to Tyr¹-glucitol GIP.
7 Figure 3C shows the prominent multiply charged species
8 $(M+3H)^{3+}$ and $(M+4H)^{4+}$ detected from the major fragment of
9 GIP at m/z 1583.8 and 1188.1, corresponding to intact M^r
10 4748.4 and 4748 Da, respectively. This corresponds
11 with the theoretical mass of the N-terminally truncated
12 form of the peptide GIP(3-42). This fragment was also
13 the major degradation product of DPP IV incubations
14 (data not shown).

15
16 Effects of GIP and Tyr¹-glucitol GIP on plasma glucose
17 homeostasis. Figures 4 and 5 show the effects of
18 intraperitoneal glucose alone (18mmol/kg) (control
19 group), and glucose in combination with GIP or Tyr¹-
20 glucitol GIP (10nmol/kg) on plasma glucose and insulin
21 concentrations. Compared with the control group,
22 plasma glucose concentrations and area under the curve
23 (AUC) were significantly lower following administration
24 of either GIP or Tyr¹- glucitol GIP (Figure 4A, B).
25 Furthermore, individual values at 15 and 30 minutes
26 together with AUC were significantly lower following
27 administration of Tyr¹-glucitol GIP as compared to GIP.
28 Consistent with the established insulin-releasing
29 properties of GIP, plasma insulin concentrations of
30 both peptide-treated groups were significantly raised
31 at 15 and 30 minutes compared with the values after
32 administration of glucose alone (Figure 5A). The
33 overall insulin responses, estimated as AUC were also
34 significantly greater for the two peptide-treated
35 groups (Figure 5B). Despite lower prevailing glucose
36 concentrations than the GIP-treated group, plasma

insulin response, calculated as AUC, following Tyr¹-glucitol GIP was significantly greater than after GIP (Figure 5B). The significant elevation of plasma insulin at 30 minutes is of particular note, suggesting that the insulin-releasing action of Tyr¹-glucitol GIP is more protracted than GIP even in the face of a diminished glycemic stimulus (Figures 4A, 5A).

Discussion

The forty two amino acid GIP is an important incretin hormone released into the circulation from endocrine K-cells of the duodenum and jejunum following ingestion of food [36]. The high degree of structural conservation of GIP among species supports the view that this peptide plays an important role in metabolism [12]. Secretion of GIP is stimulated directly by actively transported nutrients in the gut lumen without a notable input from autonomic nerves [12]. The most important stimulants of GIP release are simple sugars [37] and unsaturated long chain fatty acids [38], with amino acids exerting weaker effects [39]. As with tGLP-1, the insulin-releasing effect of GIP is strictly glucose-dependent [30,40]. This affords protection against hypoglycemia and thereby fulfils one of the most desirable features of any current or potentially new antidiabetic drug [41].

The present results demonstrate for the first time that Tyr¹-glucitol GIP displays profound resistance to serum and DPP IV degradation. Using ESI-MS the present study showed that native GIP was rapidly cleaved in vitro to a major 4748.4 Da degradation product, corresponding to GIP(3-42) which confirmed previous findings using matrix-assisted laser desorption ionization time-of-flight mass spectrometry [42]. Serum degradation was

15

1 completely inhibited by diprotin A (Ile-Pro-Ile), a
2 specific competitive inhibitor of DPP IV, confirming
3 this as the main enzyme for GIP inactivation in vivo
4 [4,5]. In contrast, Tyr¹-glucitol GIP remained almost
5 completely intact after incubation with serum or DPP IV
6 for up to 12 hours. This indicates that glycation of
7 GIP at the amino-terminal Tyr¹ residue masks the
8 potential cleavage site from DPP IV and prevents
9 removal of the Tyr¹-Ala² dipeptide from the N-terminus
10 preventing the formation of GIP(3-42).

11
12 Consistent with in vitro protection against DPP IV,
13 administration of Tyr¹-glucitol GIP significantly
14 enhanced the antihyperglycemic activity and
15 insulin-releasing action of the peptide when
16 administered with glucose to rats. Native GIP enhanced
17 insulin release and reduced the glycemic excursion as
18 observed in many previous studies [12,40]. However,
19 amino-terminal glycation of GIP increased the insulin-
20 releasing and antihyperglycemic actions of the peptide
21 by 62% and 38% respectively, as estimated from AUC
22 measurements. Detailed kinetic analysis is difficult
23 due to necessary limitation of sampling times, but the
24 greater insulin concentrations following Tyr¹-glucitol
25 GIP as opposed to GIP at 30 minutes post-injection is
26 indicative of a longer half-life. The glycemic rise
27 was modest in both peptide-treated groups and glucose
28 concentrations following injection of Tyr¹-glucitol GIP
29 were consistently lower than after GIP. Since the
30 insulintropic actions of GIP are glucose-dependent
31 [30,40], it is likely that the relative
32 insulin-releasing potency of Tyr¹-glucitol GIP is
33 greatly underestimated in the present in vivo
34 experiments.

35
36 In vitro studies in the laboratory of the present

1 inventors using glucose-responsive clonal B-cells
2 showed that the insulin-releasing potency of Tyr¹-
3 glucitol GIP was several order of magnitude greater
4 than GIP and that it's effectiveness was more sensitive
5 to change of glucose concentrations within the
6 physiological range. Together with the present *in vivo*
7 observations, this suggests that N-terminal glycation
8 of GIP confers resistance to DPP IV degradation whilst
9 enhancing receptor binding and insulin secretory
10 effects on the B-cell. These attributes of Tyr¹-
11 glucitol GIP are fully expressed *in vivo* where DPP IV
12 resistance impedes degradation of the peptide to GIP(3-
13 42), thereby prolonging the half-life and enhancing
14 effective concentrations of the intact biologically
15 active peptide. It is thus possible that glycated GIP
16 is enhancing insulin secretion *in vivo* both by enhanced
17 potency at the receptor as well as improving DPP IV
18 resistance. Thus numerous studies have shown that GIP
19 (3-42) and other N-terminally modified fragments,
20 including GIP(4-42), and GIP (17-42) are either weakly
21 effective or inactive in stimulating insulin release
22 [4,43-45]. Furthermore, evidence exists that N-
23 terminal deletions of GIP result in receptor antagonist
24 properties in GIP receptor transfected Chinese hamster
25 kidney cells [9], suggesting that inhibition of GIP
26 catabolism would also reduce the possible feedback
27 antagonism at the receptor level by the truncated
28 GIP(3-42).

29
30 In addition to its insulinitopic actions, a number of
31 other potentially important extrapancreatic actions of
32 GIP may contribute to the enhanced antihyperglycemic
33 activity and other beneficial metabolic effects of Tyr¹-
34 glucitol GIP. These include the stimulation of glucose
35 uptake in adipocytes, increased synthesis of fatty
36 acids and activation of lipoprotein lipase in adipose

tissue [46-48]. GIP also promotes plasma triglyceride clearance in response to oral fat loading [49]. In liver, GIP has been shown to enhance insulin-dependent inhibition of glycogenolysis [50]. GIP also reduces both glucagon-stimulated lipolysis in adipose tissue as well as hepatic glucose production [51]. Finally, recent findings indicate that GIP has a potent effect on glucose uptake and metabolism in mouse isolated diaphragm muscle [52]. This latter action may be shared with tGLP-1 [53,54] and both peptides have additional benefits of stimulating somatostatin secretion and slowing down gastric emptying and nutrient absorption [1,55].

In conclusion, this study has demonstrated for the first time that the glycation of GIP at the amino-terminal Tyr¹ residue limits GIP catabolism through impairment of the proteolytic actions of serum petidases and thus prolongs its half-life in vivo. This effect is accompanied by enhanced antihyperglycemic activity and raised insulin concentrations in vivo, suggesting that such DPP IV resistant analogues should be explored alongside tGLP-1 as potentially useful therapeutic agents for NIDDM. Tyr¹-glucitol GIP appears to be particularly interesting in this regard since such amino-terminal modification of GIP enhances rather than impairs glucose-dependent insulinotropic potency as was observed recently for tGLP-1 [28].

Detailed Figure Legends

Figure 1 illustrates degradation of GIP and Tyr¹-glucitol GIP by DPP IV. Representative HPLC profiles obtained after incubation of GIP (left panels) or Tyr¹-glucitol GIP exposed to DPP IV were separated on a

1 Vydac C-18 column using linear gradients of 0% to 31.5%
2 acetonitrile over 15 minutes, to 38.5% over 30 minutes
3 and from 38.5% to 70% acetonitrile over 5 minutes.
4 Left hand panels show HPLC profiles of intact GIP
5 (retention time 21.93 min) and GIP(3-42) (retention
6 time 21.61 min), Right hand panels show HPLC profiles
7 obtained for Tyr¹-glucitol GIP (retention time 21.75
8 min). HPLC peaks corresponding to intact GIP, GIP(3-
9 42) and Tyr¹-glucitol GIP are indicated.

10
11 Figure 2 illustrates degradation of GIP and Tyr¹-
12 glucitol GIP by human plasma. Representative HPLC
13 profiles obtained after incubation of GIP (left panels)
14 and Tyr¹-glucitol GIP (right panels) with human plasma
15 for 0 and 4 hours and for 4 hours in the presence of
16 5mU of diprotin A(DPA). GIP and Tyr¹-glucitol GIP
17 incubations were separated with a Vydac C-18 column
18 using linear gradients 0% to 31.5% acetonitrile over 15
19 minutes, to 38.5% over 30 minutes and from 38.5% to 70%
20 acetonitrile over 5 minutes. Peaks corresponding with
21 intact GIP, GIP(3-42) and Tyr¹-glucitol GIP are
22 indicated. A major peak corresponding to the specific
23 DPP IV inhibitor tripeptide DPA appears in the bottom
24 panels with retention time 16.29 min.

25
26 Figure 3 illustrates electrospray ionization mass
27 spectrometry of GIP, Tyr¹-glucitol GIP and the major
28 degradation fragment GIP(3-42). Samples containing
29 (A)GIP, (B)Tyr¹-glucitol GIP and (C) the major
30 degradation fragment of GIP (GIP(3-42)) isolated from
31 plasma incubations (Figure 2). Peptides were dissolved
32 (approximately 400 pmol) in 100μl of water and applied
33 to the LC/MS equipped with a microbore C-18 HPLC
34 column. Samples (30μl direct loop injection) were
35 applied at a flow rate of 0.2ml/min, under isocratic
36 conditions 35% acetonitrile/water. Mass spectra were

recorded using a quadripole ion trap mass analyzer. Spectra were collected using full ion scan mode over the mass-to-charge (m/z) range 150-2000. The molecular masses (M_r) of GIP and related structures were determined from ESI-MS profiles using prominent multiple charged ions and the following equation $M_r = iM_1 - iM_n$ (see Research Design and Methods Section).

Figure 4 - effects of GIP and glycated GIP on plasma glucose homeostasis. (A) Plasma glucose concentrations after i.p. glucose alone (18mmol/kg) (control group), or glucose in combination with either GIP or Tyr¹-glucitol GIP (10nmol/kg). The time of injection is indicated by the arrow (0 min). (B) Plasma glucose AUC values for 0-60 min post injection. Values are mean \pm SEM for six rats. ** $P < 0.01$, *** $P < 0.001$ compared with GIP and Tyr¹-glucitol GIP; † $P < 0.05$, †† $P < 0.01$ compared with non-glycated GIP.

Figure 5 - effects of GIP on plasma insulin responses (A). Plasma insulin concentrations after i.p. glucose alone (18 mmol/kg) (control group), or glucose in combination with either with GIP or glycated GIP (10nmol/kg). The time of injection is indicated by the arrow. (B) Plasma insulin AUC values were calculated for each of the 3 groups up to 90 minutes post injection. The time of injection is indicated by the arrow (0 min). Plasma insulin AUC values for 0-60 min post injection. Values are mean \pm SEM for six rats. * $P < 0.05$, ** $P < 0.001$ compared with GIP and Tyr¹-glucitol GIP; † $P < 0.05$, †† $P < 0.01$ compared with non-glycated GIP.

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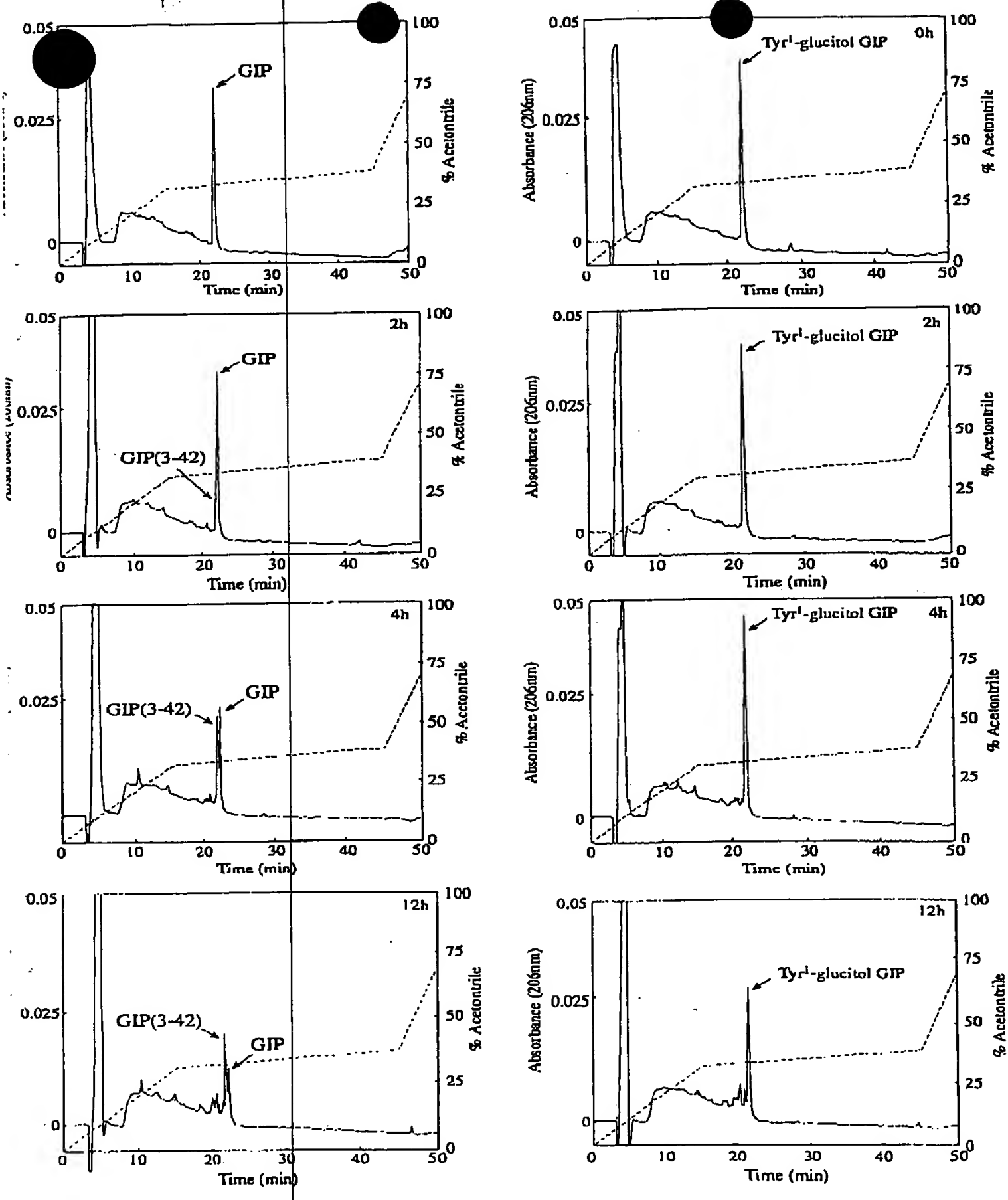
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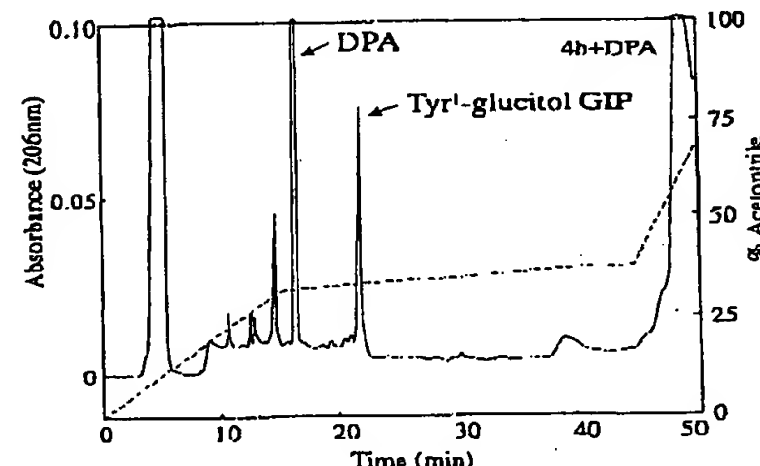
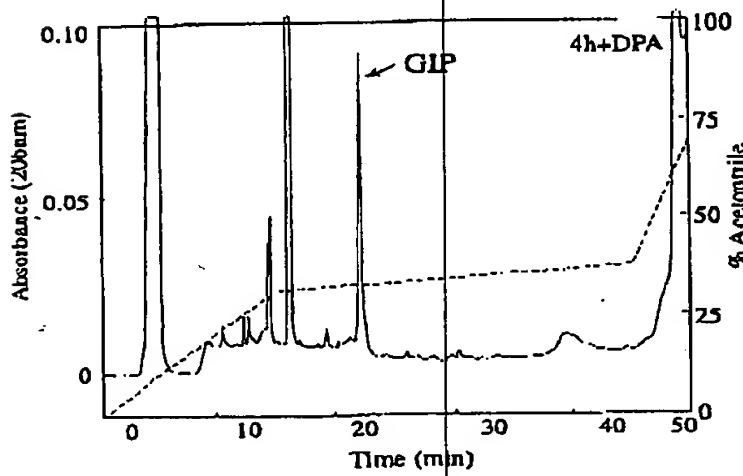
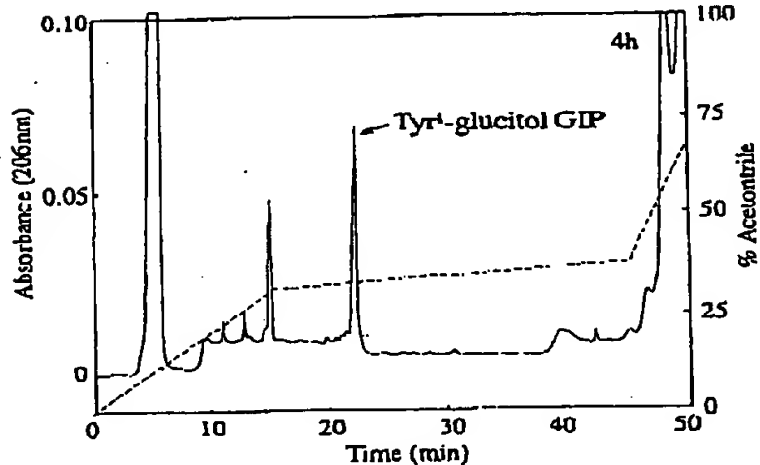
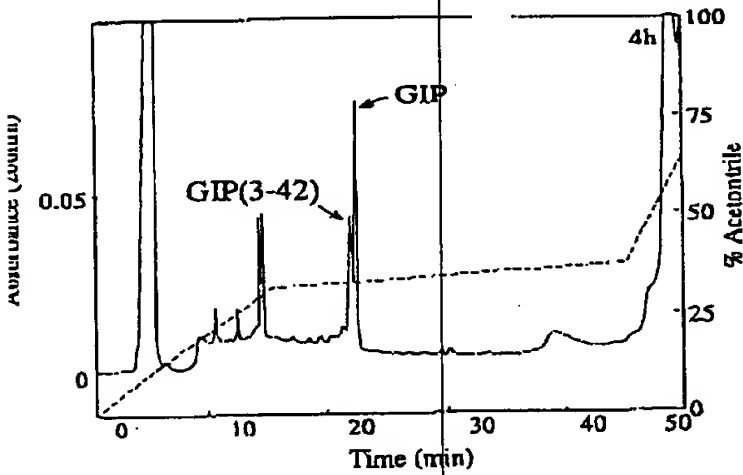
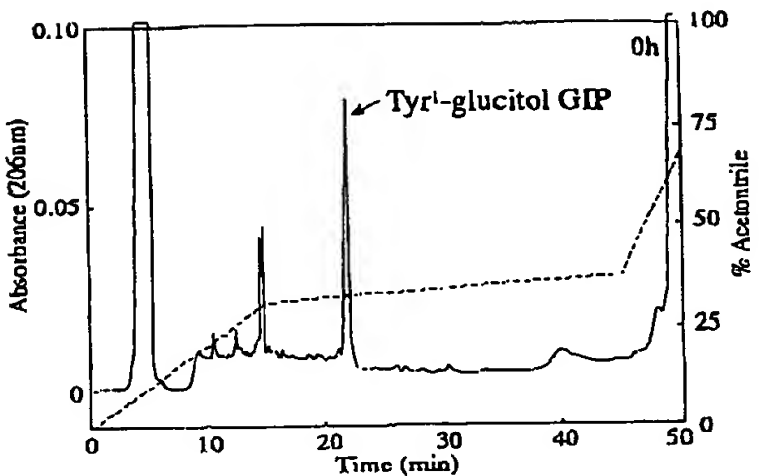
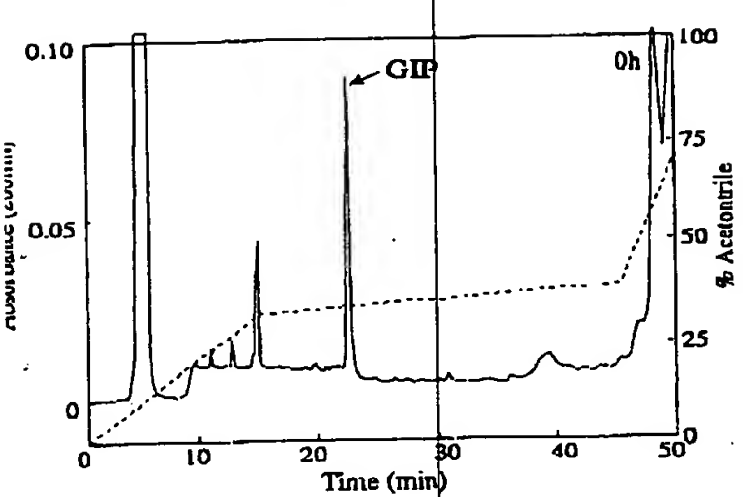
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Fig 1



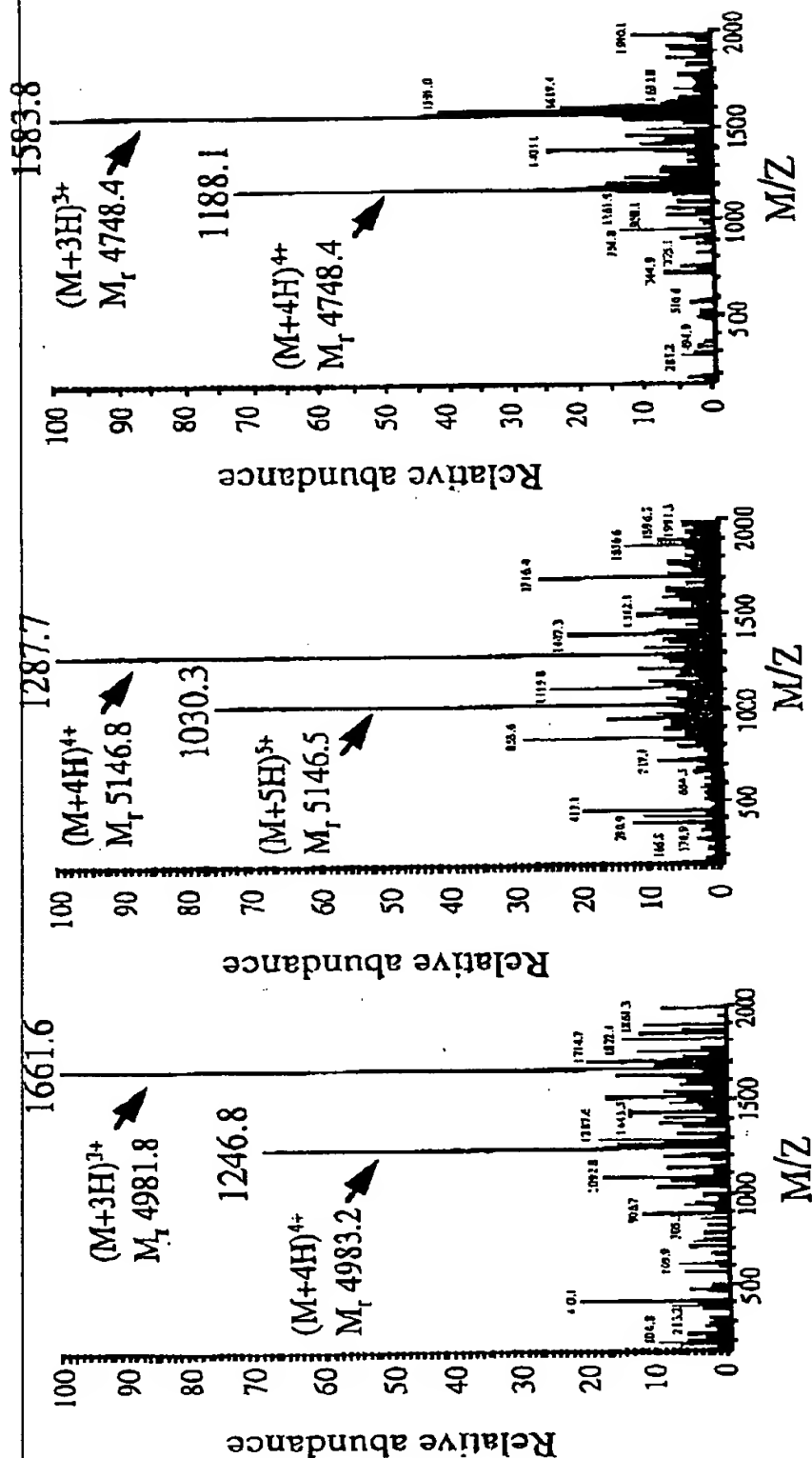
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Fig 2



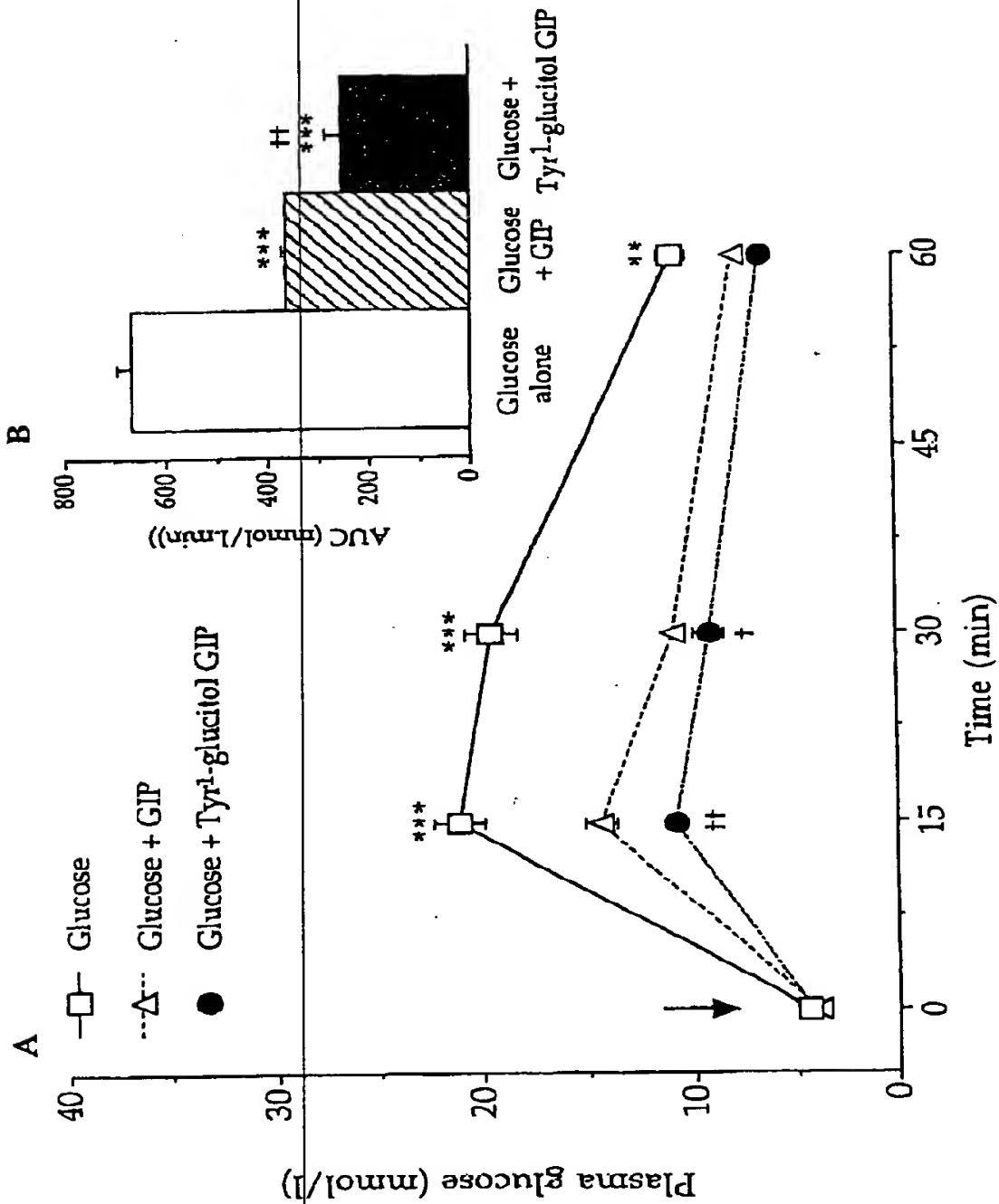
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(A) GIP (B) Tyr¹-glucitol GIP (C) GIP(3-42)



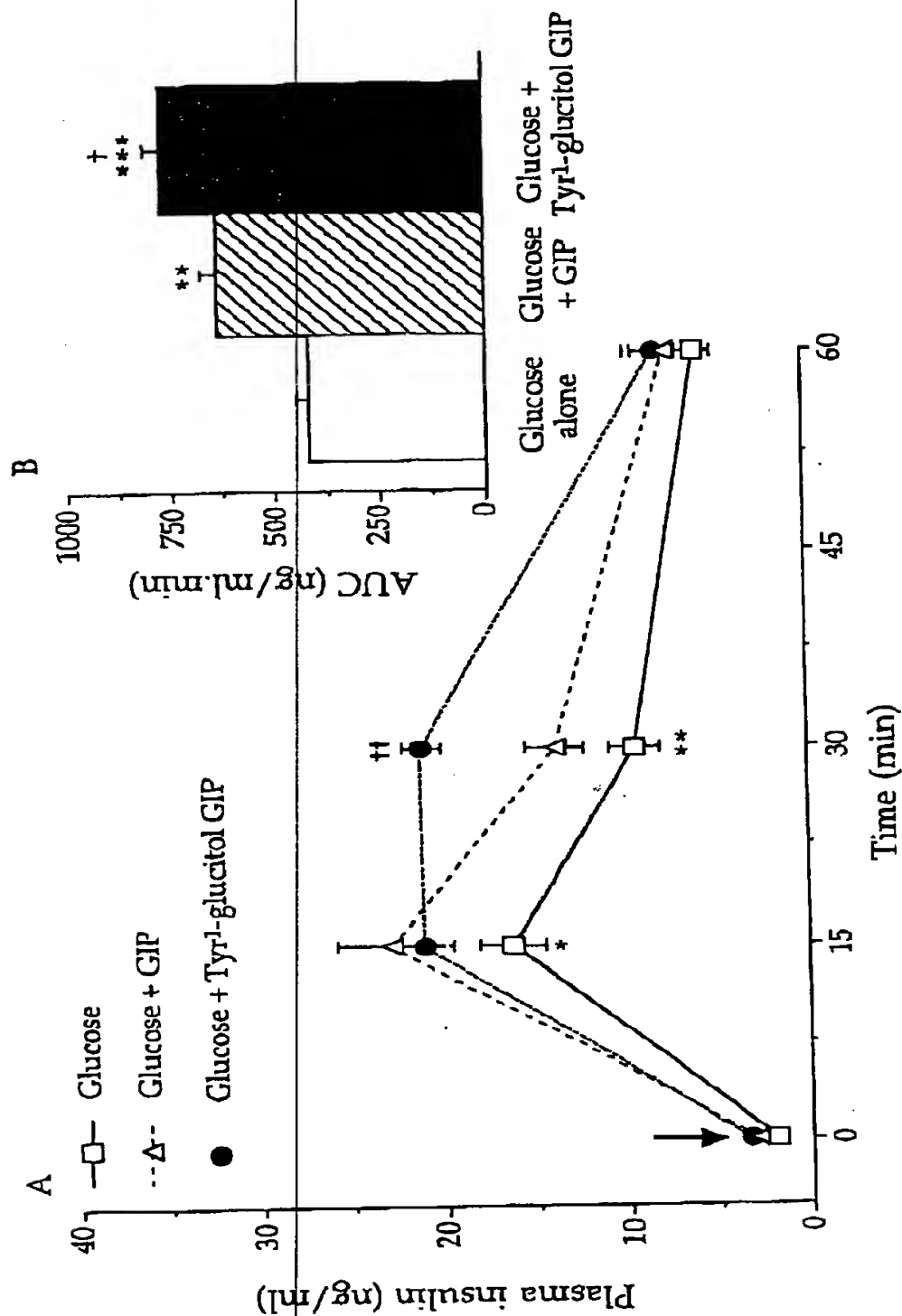
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Fig 4



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Fig 5



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